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Microscopical, Biochemical, and Immunological Studies of the Immune Defense System of the Horseshoe Crab, *Limulus polyphemus*

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Abstract. The immune defense system of *Limulus polyphemus* was explored. Two distinct cells, granulocytes and plasmatocytes, were revealed in the hemolymph by light microscopy of live cells, by light microscopy serial sectioning, and by transmission electron microscopy combined with immuno-gold labelling. The granulocytes (amebocytes) comprised about 99% of the hemocytes. They had a heterochromatic nucleus, distended but poorly developed RER, few free ribosomes, few mitochondria, but many large secretory granules. While the majority of these were uniformly stained, structured granules were also present. Monoclonal antibodies revealed that coagulogen is present in both types of large secretory granules. It is suggested that a structured granule is an immature stage leading to a uniformly stained granule. The plasmatocytes had an euchromatic nucleus, extended RER consisting of tubular or flattened cisternae, many free ribosomes, many mitochondria, but few, if any, large secretory granules. Coagulogen was not detected in plasmatocytes. Following immunizations of five rabbits for more than one year with cell-free hemolymph, 14 polypeptides were immunogenic in rabbits as visualized by crossed immunoelectrophoresis. However, O'Farrell gel electrophoresis combined with silver staining identified more than 70 major polypeptides in the cell-free hemolymph. At least 60 of these had pI-values between 7.5 and 5.5, and Mr-values from larger than 200×10^3 to smaller than 21×10^3.

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Introduction

The four recognized extant species of horseshoe crabs are "living fossils," whose morphology has remained unchanged for about 200 million years (Sekiguchi and Sugita, 1980). If this stability is reflected in their physiology, studies of their immune defense system may reveal some of the molecular mechanisms responsible for their success in an environment of gram negative bacteria (Bang, 1956). Furthermore, such studies may eventually facilitate our understanding of how the immune defense system evolved in higher and more recent phyla than horseshoe crabs.

Introduction of gram negative bacteria into the hemolymph of *Limulus polyphemus* results in clotting (Bang, 1956). This coagulation is a result of extensive exocytosis from the hemocytes (Levin and Bang, 1964). In intact

Figure 1. Live granulocyte (A) and plasmatocyte (B) from *Limulus polyphemus* examined by light microscopy interference contrast optics immediately after sampling. Nucleus (N).

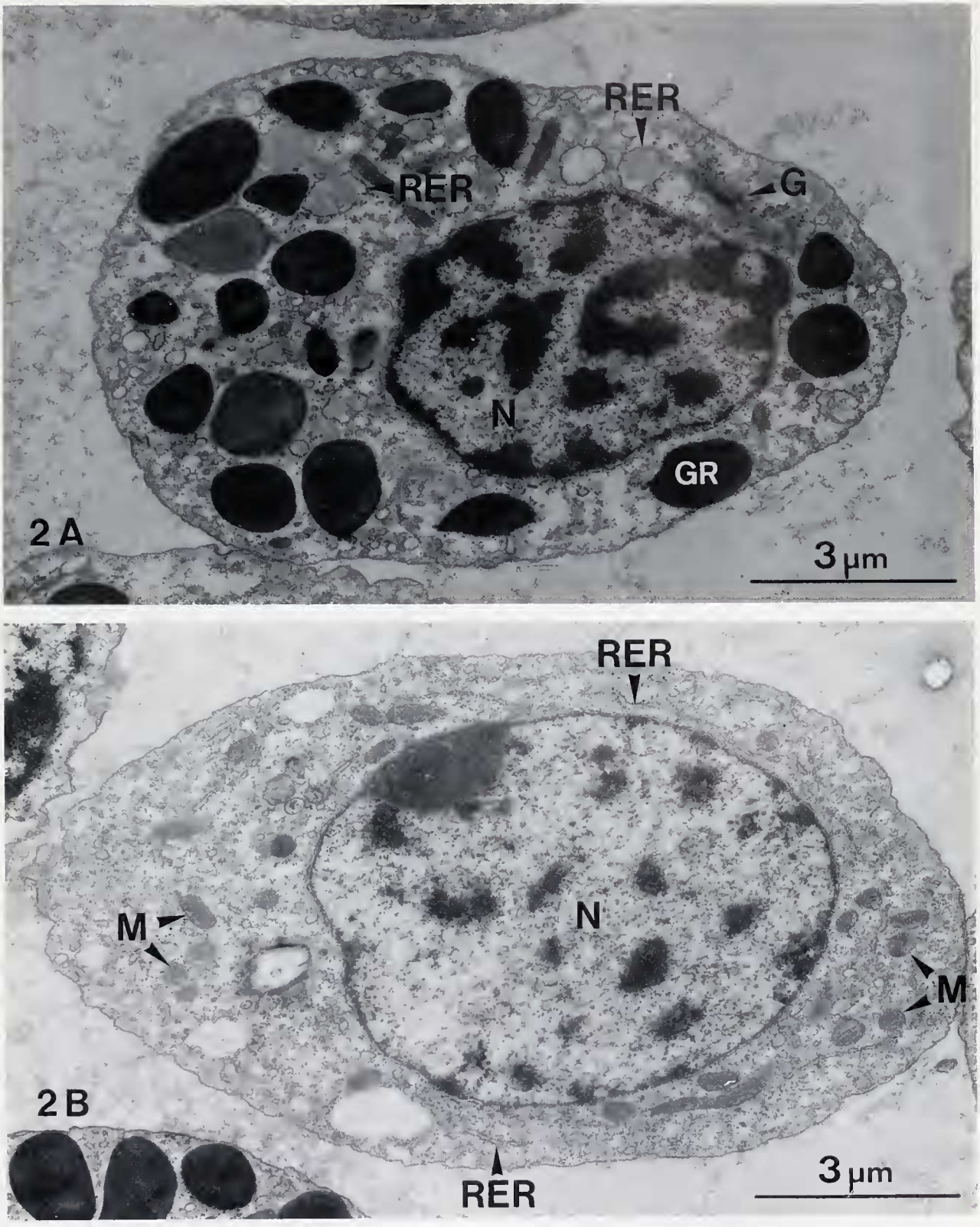
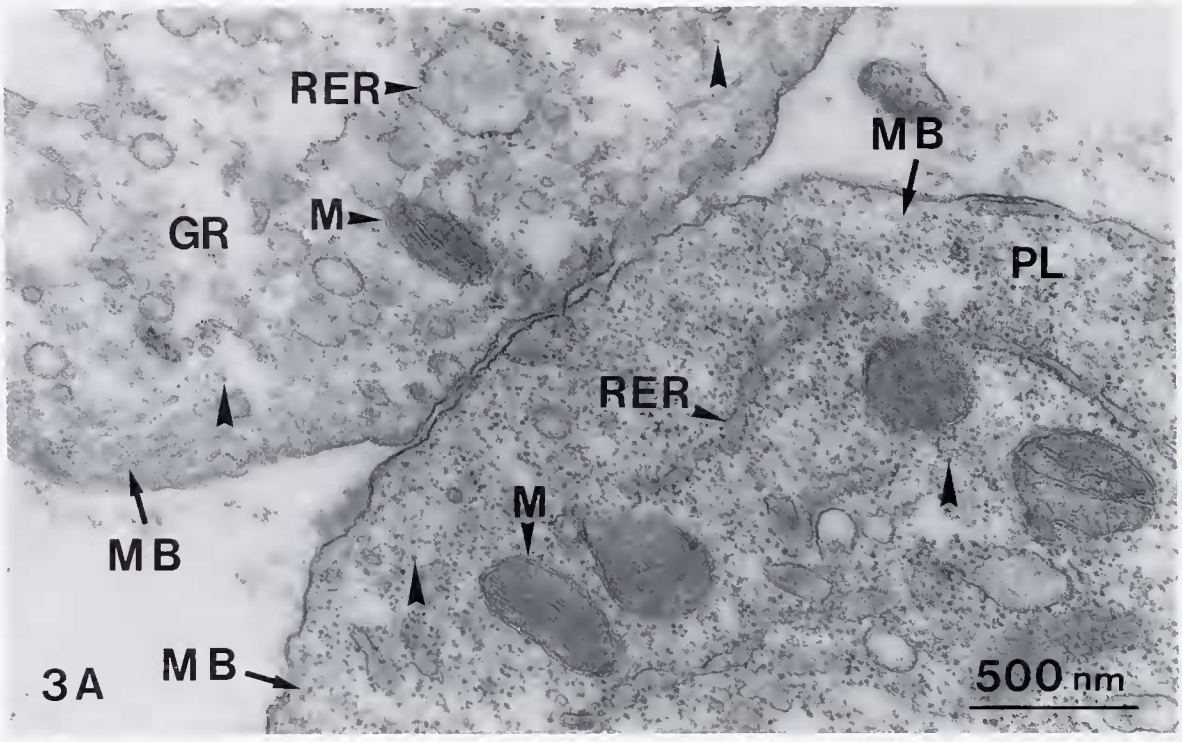
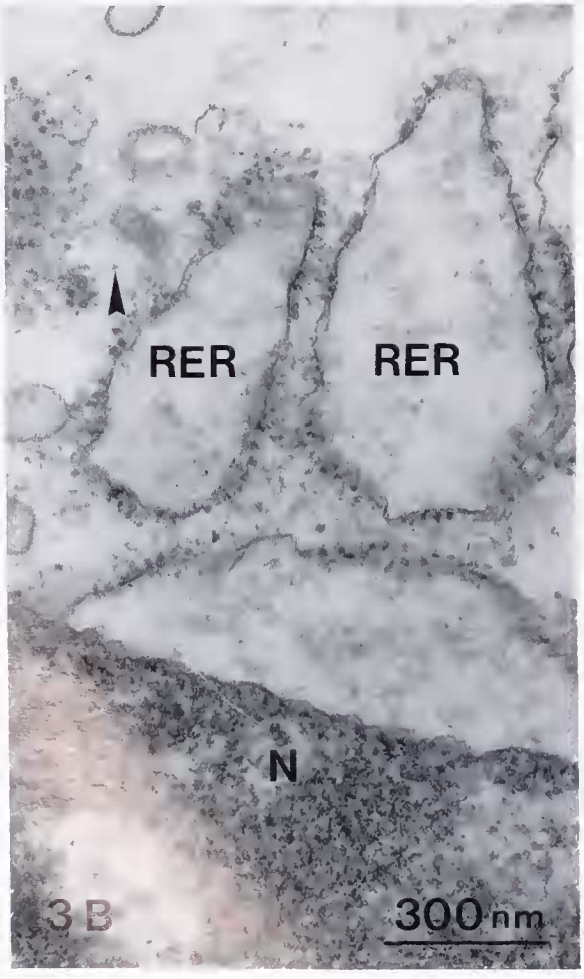
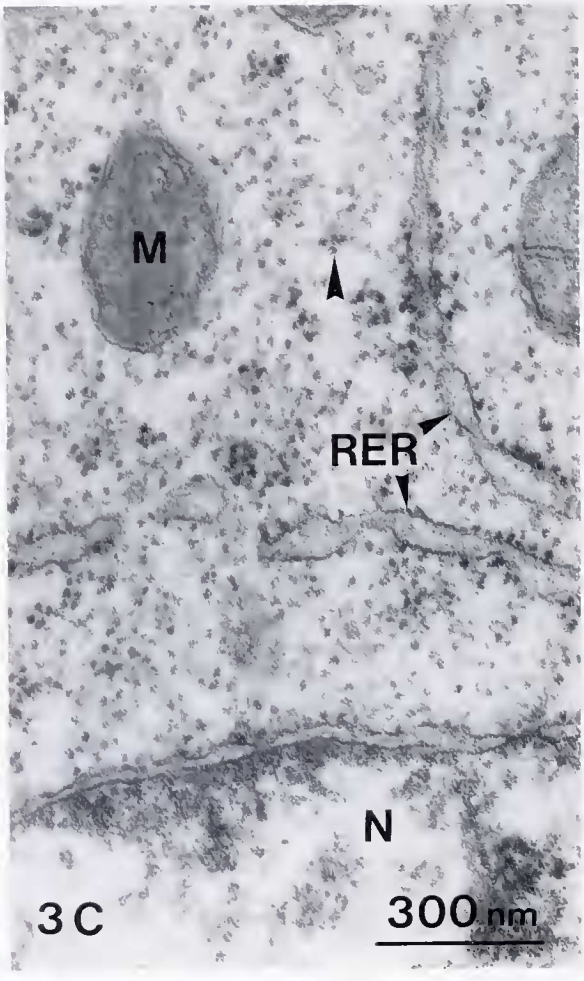


Figure 2. Granulocyte (A) and plasmatocyte (B) from *Limulus polyphemus* examined by TEM. Golgi complex (G), large uniform dense secretory granule (GR); mitochondria (M); nucleus (N); rough endoplasmic reticulum (RER).


RER
MB
GR
M
PL
RER
M
MB
MB
3A
500 nm


RER
RER
N
3B
300 nm


M
RER
N
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animals a tiny fraction of the hemocytes may be secreting spontaneously, while stimulation with gram negative bacteria or their lipopolysaccharides (LPS) induces a burst of exocytosis in the secretory cells (Armstrong, 1979; Ornberg, 1985; Iwanaga *et al.*, 1986). Lysates of hemocytes are now extensively used to detect minute quantities of LPS (Watson *et al.*, 1987).

The horseshoe crabs *L. polyphemus* and *Tachypleus tridentatus* have been reported to contain a single and two granular hemocytes, respectively (Dumont *et al.*, 1966; Shishikura *et al.*, 1977; Shishikura and Sekiguchi, 1979; Copeland and Levin, 1985). Cyanoblasts and cyanocytes are also evident in the general circulation of *L. polyphemus* (Fahrenbach, 1970; Sherman, 1981). They are characterized by their large size (diameter up to 100 μm) and their content of cytoplasmic hemocyanin crystals.

Recently we investigated the fine structure of unstimulated hemocytes from *T. tridentatus* using transmission electron microscopy (TEM) and light microscopy (LM) serial sectioning (P. P. Jakobsen, and P. Suhr-Jessen, unpub.). We found two populations of cells: granulocytes with a heterochromatic nucleus, many large secretory granules, and a poorly developed RER; and plasmatocytes with an euchromatic nucleus, few large secretory granules, and a well-developed RER. Cyanoblasts or cyanocytes were not observed. These observations prompted us to reinvestigate *L. polyphemus*, and here we report on the presence of granulocytes and plasmatocytes in this species too.

Few polypeptides have so far been discovered in the cell-free hemolymph. They include hemocyanin, an alfa 2-macroglobulin-like protease inhibitor, hemagglutinins, and lectins (Nachum *et al.*, 1979; Pistole, 1979; Cohen *et al.*, 1984; Pistole and Graf, 1984; Kempter *et al.*, 1985; Armstrong, 1985; Aketagawa, 1986; Liu *et al.*, 1987). Two-dimensional gel-electrophoresis combined with silver staining has previously identified an unforeseen high number of plasma and cerebrospinal fluid polypeptides in other organisms (Anderson and Anderson, 1977; Merrill *et al.*, 1981; Suhr-Jessen and Rasmussen, 1988). In *L. polyphemus*, these methods reveal more than 70 polypeptides in the cell-free hemolymph, among which only 14 are immunogenic in rabbit.

Materials and Methods

Animals

L. polyphemus (prosoma width: 4–22 cm) was purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts, and kept at 15°C in The Salt Water Aquarium of Funen in seawater or in artificial seawater containing 3.0% NaCl. Hemolymph was drawn by cardiac puncture at the ethanol cleaned prosoma-opistosoma junction using a 19 gauge needle alone or combined with a 5 ml syringe. Cell-free hemolymph was prepared as described (Tvede and Baek, 1983). Samplings from all animals gave similar results.

Light microscopy of live cells

One part hemolymph was instantly diluted into one volume endotoxin free 3% NaCl containing 2 m*M* propranolol (Murer *et al.*, 1975). Samples were placed between alcohol cleaned object and cover glasses, examined using interference contrast optics and immersion oil (Leitz; 400×), and photographed.

Light- and transmission electron microscopy of dead cells

One part hemolymph was instantly mixed with 4 parts 5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4 for 30 min at room temperature followed by 90 min at 4°C, post fixed with 2% OsO_4, contrasted with uranyl acetate, and embedded in araldite (Willumsen *et al.*, 1987). Samples were sectioned on a Reichert OM U2 ultramicrotome. For light microscopy (LM), sections (about 500 nm) were stained with toluidine blue and examined in a Leitz microscope at 400×. For transmission electron microscopy (TEM), sections (about 50 nm) were mounted on pioloform F-50 coated Ni-grids, contrasted with 0.4% lead citrate in water for 5 min, and examined in a Jeol JEM-100CX electron microscope at 80 kV.

Immuno gold labelling

One part hemolymph was instantly mixed with 4 parts 2.5% glutaraldehyde plus 3.75% paraformaldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4, and left for 60

Figure 3. A comparison of granulocytes and plasmatocytes from *Limulus polyphemus*. (A) Granulocyte (GR) and plasmatocyte (PL). Free ribosome (arrow head); marginal band of microtubules (MB); mitochondria (M); rough endoplasmatic reticulum (RER). (B) Part of granulocyte with the heterochromatic nucleus, distended RER and few free ribosomes. Nucleus (N), otherwise symbols as in A. (C) Part of plasmatocyte with the euchromatic nucleus, many free ribosomes, and flattened or tubular RER. Symbols as in B.

Table I

A comparison of the abundance of mitochondria in plasmatocytes (PL) and granulocytes (GR) determined in three independent, complete serial sections

Serial section	Number of mitochondria in Plasmatocyte	Granulocyte	Ratio PL/GR
# 1	203	61	3.33
# 2	364	120	3.03
# 3	318	114	2.79
Total	885	295	3

min at room temperature. It was then washed in cacodylate buffer (4 × 5 min), embedded in 2% agar, dehydrated in ethanol at gradually decreasing temperatures, and embedded in Lowicryl K4M according to manufacturer's guide. Formation of the ultra pyramid, sectioning, and incubation with antibodies were made the same day, since prolonged exposure to air seems to inactivate the antigens.

Incubations with antibodies were made by successively floating the grids on drops of (1) swine pre-immune serum (15 min), (2) monoclonal mouse anti-coagulogen antibody (120 min), (3) 0.05 *M* Tris buffer, pH 7.4 (3 × 5 min), (4) swine anti-mouse antibodies coupled to 10 nm gold particles (DAKOPATTS, 60 min), and (5) Tris buffer as above. While pre-immune serum was used directly, anti-coagulogen and gold-coupled antibodies were immediately diluted 50 fold with Tris buffer in the absence and presence of 2% fish gelatine (Sigma), respectively (Behnke *et al.*, 1986), before use. Samples were then contrasted in 1% uranyl acetate (10 min) followed by 0.4% lead citrate (5 min), and examined by TEM as described above.

Antibody production

To generate polyclonal antibodies against cell-free hemolymph, five rabbits were injected intradermally with 100 μl of cell-free hemolymph and an equal volume of Freunds incomplete adjuvant (Harboe and Ingild, 1973). The first 5 injections were spaced 14 days apart, and they were followed by monthly booster vaccinations for 6 months before the first bleeding. Thereafter, the immunization continued for more than 12 months. One week after each vaccination, 40 ml of blood was sampled from each rabbit. Serum from the five rabbits were pooled, and immunoglobulin G was purified and concentrated (Harboe and Ingild, 1973).

The generation of mouse monoclonal antibodies against coagulogen is described elsewhere (Zhang *et al.*, 1988).

Crossed immunoelectrophoresis

The immunoelectrophoresis was performed on 100 × 100 mm gels according to Weeke (1973). Five μl cell-free hemolymph was separated in the first dimension. The second dimension contained polyclonal rabbit antibodies against cell-free hemolymph (5 μl/cm^2 gel).

Polyacrylamide gel electrophoresis

Freshly drawn hemolymph was immediately centrifuged (5.000 g/4 min/20°C). The upper two thirds of the cell-free hemolymph was quickly diluted into five volumes of fresh lysis buffer (5 m*M* Tris pH 6.8, 2.5% SDS, 10% 2-mercaptoethanol, 1 m*M* PMSF), and boiled 4 min. Samples were then dialyzed at 4°C against double-distilled water, and freeze-dried. Lyophilized cell-free hemolymph was separated on two-dimensional 1 mm 10–16% polyacrylamide gradient gels and silver stained (O'Farrell, 1975; Merrill *et al.*, 1981; Suhr-Jessen *et al.*, 1986). This allowed the detection of less than 1 ng of each of the molecular weight markers myosin, beta-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Mr-values of 200, 116, 92, 66, 45, 31, 21.5, and 14.4 × 10^3, respectively).

Results

General morphology of hemocytes

The hemocytes of *L. polyphemus* were spheroid and usually 15–20 μm at their longest axis (Figs. 1, 2, and 5). They contained a single non-lobated nucleus with one or a few nucleoli, RER, Golgi-complexes, mitochondria, and a marginal band of microtubules. Centrioles were present, but no signs of mitosis were seen.

Table II

A comparison of the main differences between plasmatocytes and granulocytes

	Plasmatocyte	Granulocyte
Nucleus	euchromatic	heterochromatic
RER	flattened and well developed	distended but poorly developed
Free ribosomes	many	few
Large secretory granules	few—if any	many
Coagulogen	no	yes
Mitochondria	many	few
Frequency	1%	99%

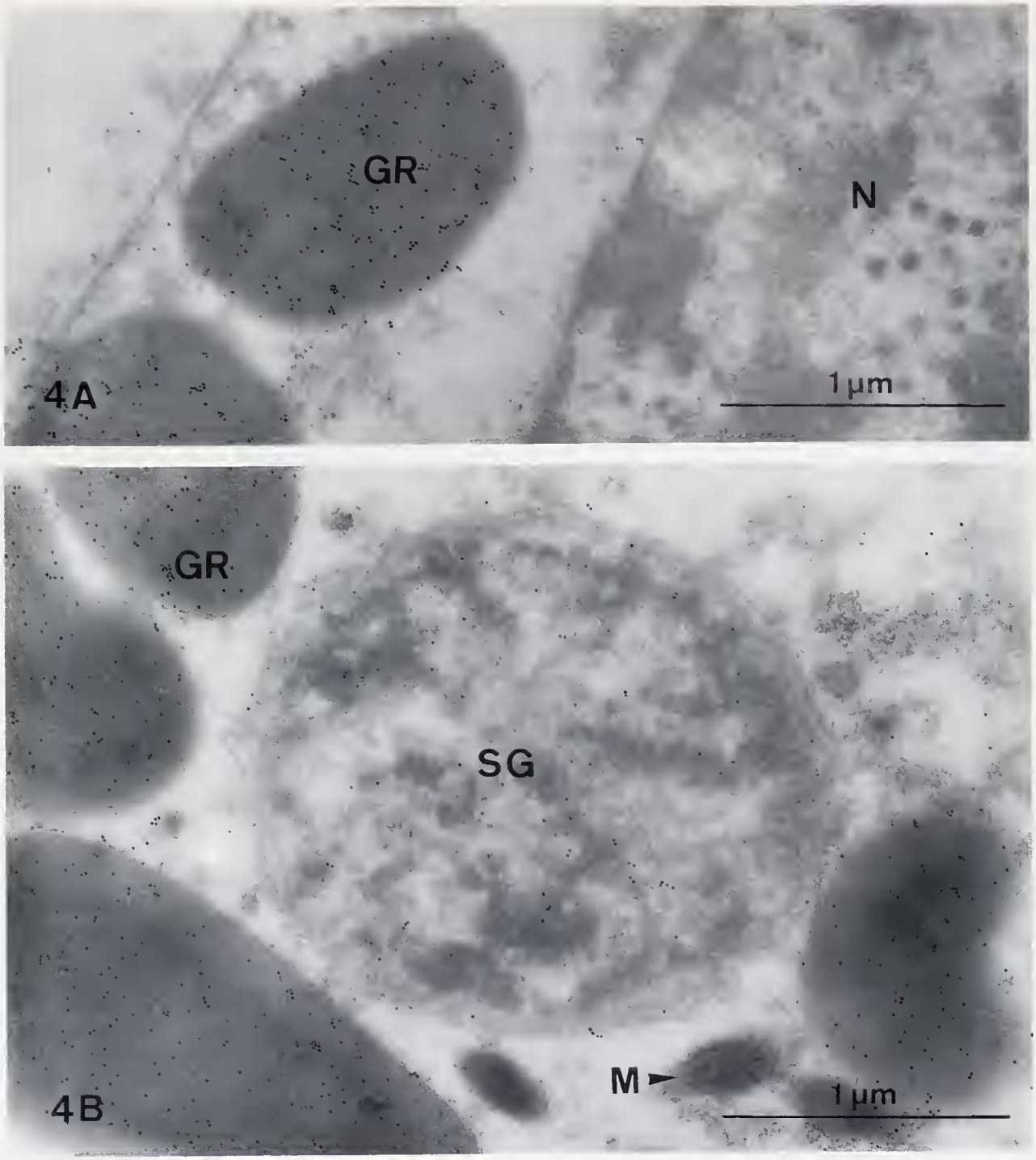
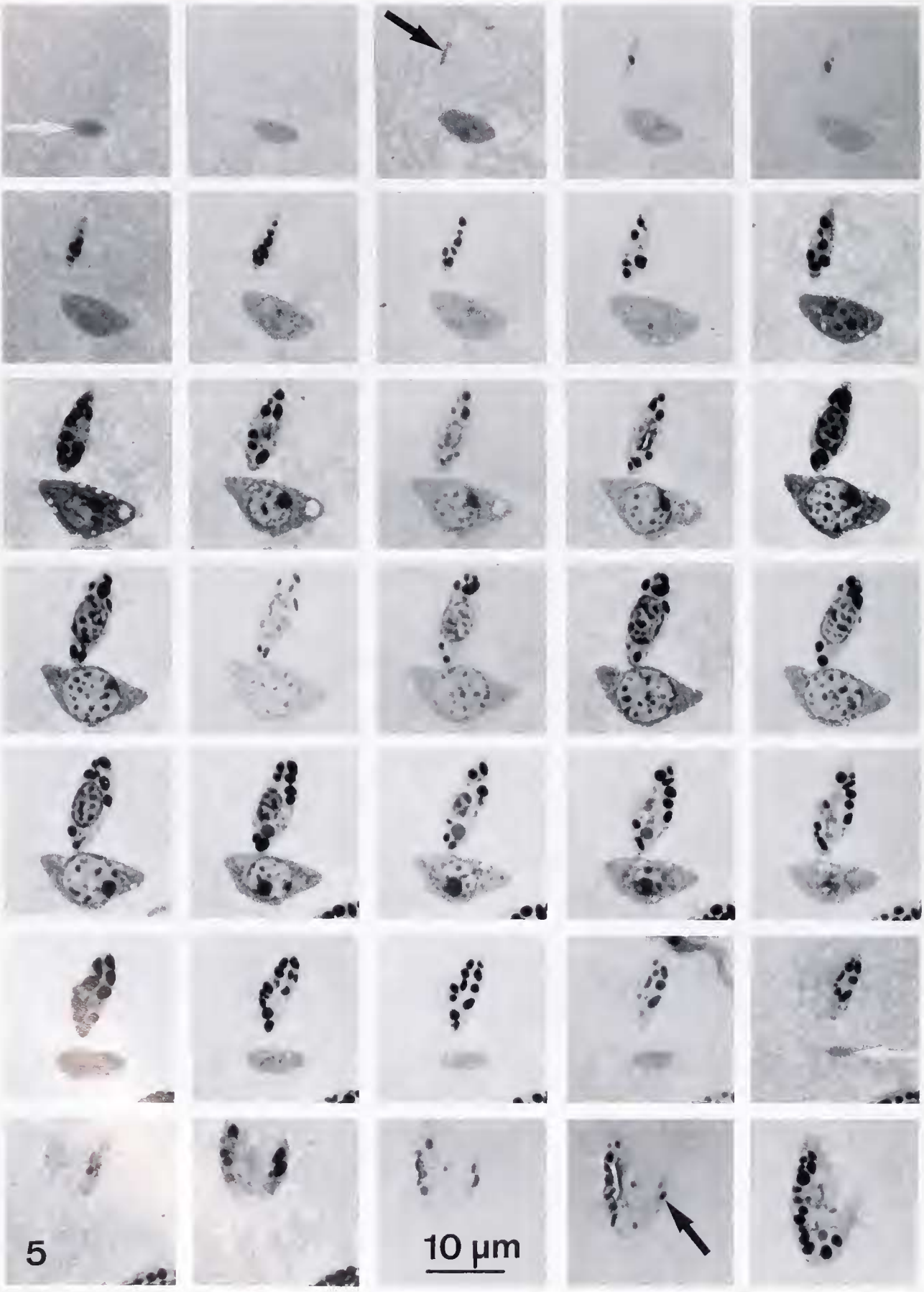


Figure 4. *Limulus polyphemus* granulocyte embedded in Lowicryl and incubated with monoclonal antibodies against coagulogen followed by gold-coupled secondary antibodies. Uniform secretory granules (GR) and structured granules (SG) are labelled. Mitochondria (M); nucleus (N).

Granulocytes

Granulocytes comprised about 99% of the hemocytes. They had a heterochromatic nucleus, a distended but poorly developed RER, few free ribosomes, few mitochondria, but many large secretory granules (Figs. 1–3, and 5). The content of the majority of the large granules is uniform, but structured


5
10 µm

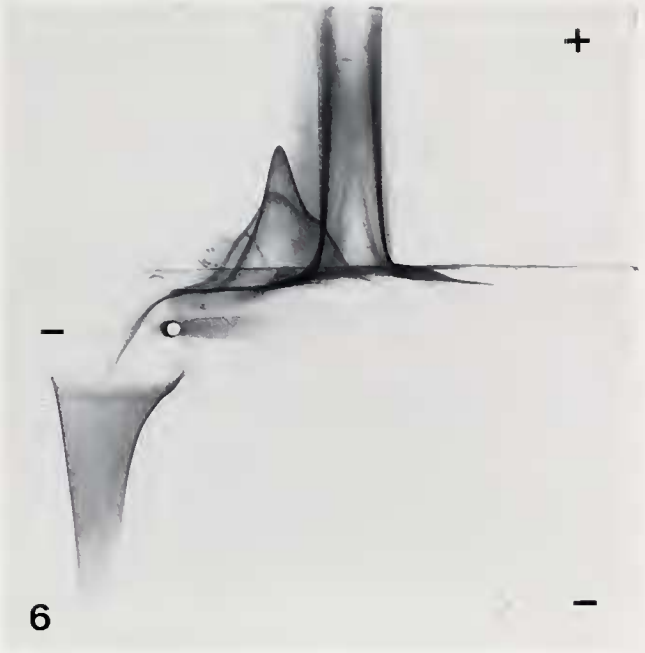


Figure 6. Crossed immunoelectrophoresis of cell-free hemolymph from *Limulus polyphemus*. The second dimension contained polyspecific rabbit antibodies against cell-free hemolymph.

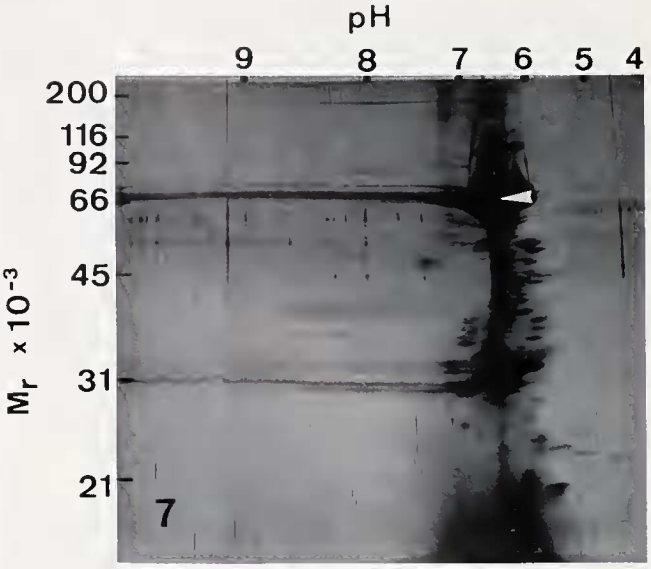


Figure 7. Cell-free hemolymph from *Limulus polyphemus* separated by O'Farrell gel electrophoresis and silver stained. Hemocyanin (arrow head).

granules are also present (Fig. 4; Dumont *et al.*, 1966; Copeland and Levin, 1985).

Plasmatocytes

Plasmatocytes were observed in all 26 *Limulus polyphemus* examined whether young or old, male or female, immediately after shipment or following more than one year in captivity. Plasmatocytes had a large, euchromatic nucleus surrounded by flattened or tubular cisternae of RER, many free ribosomes, many mitochondria, and some digestive vacuoles (Figs. 1–3). Complete serial sections of four plasmatocytes were examined by light microscopy (Fig. 5). The total number of large secretory granules observed in these cells were zero, zero, two, and two, respectively. In contrast, serial sections of hundreds of granulocytes did not reveal sections with the characteristics of a plasmatocyte (Fig. 5).

The abundance of mitochondria

The total numbers of mitochondria were counted in three independent sets of serial sections each including one granulocyte and one plasmatocyte (Table I). Plasmatocytes contain approximately three times as many mitochondria as granulocytes.

Localization of coagulogen

To reveal the ultrastructural location of coagulogen, Lowicryl embedded hemocytes were incubated with monoclonal antibodies against coagulogen followed by gold-coupled secondary antibodies (Fig. 4). In granulocytes, gold particles crowded all over the uniform secretory granules, while the number of gold particles increased with the amounts of material present in the structured granules. The density of gold particles seen in areas outside the granules is low and equivalent to background. In contrast, all 10 examined plasmatocytes with a large granule did not bind immuno-gold particles.

Characterization of polypeptides from cell-free hemolymph

The *L. polyphemus* hemolymph polypeptides immunogenic in rabbits were revealed by crossed immunoelectrophoresis (Fig. 6): the 14 polypeptides distribute themselves in 3 classes with intermediate-, low-, and cathodic mobility, respectively. The intermediate mobility group is dominating in amounts and consists mainly of hemocyanin (data not shown). The cathodic migrating group

Figure 5. Light microscopy serial sections of a plasmatocyte (white arrows) and a granulocyte (black arrows). Arrows indicate the first and last section of each cell.

is not contaminating coagulogen (Baek, 1983; Baek *et al.*, 1985).

To obtain an alternative estimate of the total number of polypeptides present in the hemolymph of *L. polyphemus*, cell-free hemolymph was separated on O'Farrell gels and visualized by silver staining (Fig. 7). Among the more than 70 polypeptides detected, the most abundant (hemocyanin monomers) had a pI around 6.3 and an Mr about 60×10^3, and at least 60 others had pI-values between 7.5 and 5.5. The hemolymph polypeptides range in size from Mr-values larger than 200×10^3 to smaller than 21×10^3.

Discussion

Granulocytes and plasmatocytes

We observed two kinds of circulating spheroid hemocytes in *L. polyphemus* with many and very few, if any, secretory granules, respectively (Figs. 1–3, and 5). Their main differences are summarized in Table II. The granular cells (amebocytes) are named granulocytes, while the almost agranular cells are named plasmatocytes in agreement with the terminology for other chelicerata and for insects (Gupta, 1979; Sherman, 1981; Gupta, 1985).

The granulocyte constitutes about 99% of the cells in the present study and has previously been characterized at the TEM level (Dumont *et al.*, 1966; Murer *et al.*, 1975; Nemhauser *et al.*, 1980; Ornberg and Reese, 1981; Armstrong, 1985; Copeland and Levin, 1985).

The plasmatocyte has hitherto been overlooked in *L. polyphemus* (Sherman, 1981). Its morphology is similar to the plasmatocyte recently discovered in *T. tridentatus* by LM serial sections and TEM (P. P. Jakobsen, and P. Suhr-Jessen, unpub.). Plasmatocytes are neither granulocytes having exocytosed during sampling nor cyanoblasts or cyanocytes. Plasmatocytes have the smooth ellipsoidal shape with a marginal band characteristic of the unstimulated granulocyte in contrast to the pseudopodial form following exocytosis (Dumont *et al.*, 1966; Armstrong, 1980; Armstrong and Rickles, 1982; Armstrong, 1985). Plasmatocytes all have the same size, are present in the general circulation of all animals studied at all times, and we have been unable to detect the cytoplasmic crystals normally seen in cyanoblasts and cyanocytes (Fahrenbach, 1970; Sherman, 1981). Immunogold labellings revealed coagulogen in granulocytes but not in plasmatocytes, suggesting that they have different cell lineages. This suggestion is supported by the higher number of mitochondria and free ribosomes in plasmatocytes than in granulocytes, and by morphological differences in RER and the nucleus (Figs. 2, 3). However, plasmatocytes may be granulocytes recovering from spontaneous exocytosis so early prior to harvest of the hemolymph that the marginal band of microtubules have reformed. Since the production of granulocytes in *L. polyphemus* is not continuous (Cohen, 1985), this latter interpretation implies either (1) that approximately 1% of the hemocytes in all horseshoe crabs examined in the present study are constantly recovering from spontaneous exocytosis, and that the transition from plasmatocyte to granulocyte is so fast that the frequency of intermediate hemocytes is at least one order of magnitude lower than that of plasmatocytes, or (2) that approximately 1% of the hemocytes in each animal recover from a single burst of exocytosis long before the first sampling and that this recycling is blocked at the plasmatocyte stage. It is presently unknown if the smaller fraction of plasmatocytes seen in the present study [1% compared to 3% in *T. tridentatus* (P. P. Jakobsen and P. Suhr-Jessen, unpub.)] reflects species specific differences.

Large secretory granules

Monoclonal antibodies detected coagulogen in the uniform granules and to a lesser extent in structured granules of granulocytes (Fig. 4). Together with the finding that structured granules almost always were found in close proximity to Golgi complexes, this suggests that the structured granule is an immature stage of the uniform secretory granule as suggested by Copeland and Levin (1985).

Hemolymph polypeptides

Hemocyanin is the most abundant polypeptide in the hemolymph of horseshoe crabs (Fernandez-Moran *et al.*, 1966; Armstrong 1985). In agreement with previous studies (Bancroft *et al.*, 1966), we observe that the monomers have a pI around 6.3 and an Mr around 60×10^3 (Fig. 7). After more than 1 year of immunizations, 14 hemolymph polypeptides are immunogenic in rabbit. However, the total number of hemolymph polypeptides is several fold larger (Fig. 6). This agrees with observations from the blue mussel and from humans (Anderson and Anderson, 1977; Suhr-Jessen and Rasmussen, 1988).

The immune defense system

Hemocytes and hemolymph polypeptides constitute the immune defense system in horseshoe crabs (Ratcliffe *et al.*, 1985). We have revealed a new hemocyte, the plasmatocyte, and identified a hitherto unforeseen high number of polypeptides in the hemolymph of *L. polyphemus*. It is tempting to speculate that granulocytes and plasmatocytes operate together, and with the hemolymph polypeptides, to recognize and destroy invading

microorganisms. The techniques used in this study allow us to identify qualitative and quantitative changes in the hemolymph during and following recovery from infections, and in combination with immunoblotting to determine the specificity of the different polypeptides. Such studies may not only shed light on host-parasite interactions in horseshoe crabs and in invertebrates in general, but may also lead to improvements of the *Limulus* amebocyte lysate.

Acknowledgments

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